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Cad74A is regulated by BR and is required for robust dorsal appendage formation in *Drosophila* oogenesis

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ABSTRACT

Drosophila egg development is an established model for studying epithelial patterning and morphogenesis, but the connection between signaling pathways and egg morphology is still incompletely understood. We have identified a non-classical cadherin, *Cad74A*, as a putative adhesion gene that bridges epithelial patterning and morphogenesis in the follicle cells. Starting in mid-oogenesis, *Cad74A* is expressed in the follicle cells that contact the oocyte, including the border cells and most of the columnar follicle cells. However, *Cad74A* is repressed in two dorsolateral patches of follicle cells, which participate in the formation of tubular respiratory appendages. We show genetically that *Cad74A* is downstream of the EGFR and BMP signaling pathways and is repressed by the Zn-finger transcription factor Broad. The correlation of *Cad74A* repression in the cells that bend out of the plane of the follicular epithelium is preserved across *Drosophila* species and mutant backgrounds exhibiting a range of eggshell phenotypes. Complete removal of *Cad74A* from the follicle cells causes defects in dorsal appendage formation. Ectopic expression of Cad74A in the roof cells results in shortened, flattened appendages due to the hindered migration of the roof cells. Based on these results, we propose that *Cad74A* is part of the adhesive machinery that enables robust dorsal appendage formation, and as such provides a link between the patterning of the follicle cells and eggshell morphogenesis.

Introduction

The transformation of epithelial sheets into complex three-dimensional structures is a key process in metazoan development. Epithelial morphogenesis is often preceded by the establishment of spatially nonuniform expression patterns of genes involved in the control of adhesion and cytoskeleton architecture. These patterns then guide the coordinated cell shape changes, movements, and rearrangements that lead to the formation of three-dimensional organs (Martinez Arias and Stewart, 2002). *Drosophila* oogenesis is an excellent model for studying epithelial patterning and morphogenesis (Berg, 2005; Horne-Badovinac and Bilder, 2005; Wu et al., 2008). In particular, patterning of the follicle cells (FCs), which form an epithelial monolayer encapsulating the developing egg, leads to the formation of several eggshell structures including: a micropyle for sperm entry, an operculum that provides an escape hatch for emerging larvae, and two dorsal appendages (DAs) that serve as respiratory tubes (Hinton, 1969). The DAs form by evagination, cell shape changes and the rearrangements of two symmetric groups of FCs (Dorman et al., 2004; Horne-Badovinac and Bilder, 2005; Ward and Berg, 2005).

The roof of each DA is derived from a dorsolateral patch of follicle cells (henceforth, roof cells) that strongly express Broad (BR), a Zn-finger transcription factor (Deng and Bownes, 1997; Tzolovsky et al., 1999) Similarly, the floor of each appendage is derived from adjacent cells (floor cells) that express *rhomboid* (*rho*), a gene encoding an intracellular protease in the epidermal growth factor receptor (EGFR) pathway (Deng and Bownes, 1997; Ruohola-Baker et al., 1993; Ward et al., 2006). The signaling and transcriptional mechanisms that establish the precise expression patterns of BR and *rho* have become progressively characterized (Dorman et al., 2004; Ward and Berg, 2005; Ward et al., 2006; Yakoby et al., 2008). Furthermore, the morphogenetic steps leading to DA formation have been carefully dissected (Dorman et al., 2004). In particular, the initial stage of DA formation begins with the apico-basal elongation of the DA primordia and the apical constriction and cell intercalation of the roof cells. As the roof cells bend out of the plane of the follicular epithelium, the floor cells slip underneath the roof cells to seal the bottom of the DAs, thereby forming tubular structures.

Elucidating the mechanisms of epithelial morphogenesis requires the identification of genes directly involved in the control of cell

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shape, adhesion, and motility. In other models of epithelial morphogenesis, cadherin proteins have been shown to play an important role in regulating cell–cell adhesion, cell rearrangements, and cell structure (Tepass, 1999). For example, previous studies established that DE-cadherin mediates cell sorting and cell migration events during oogenesis (Godt and Tepass, 1998; Gonzalez-Reyes and St Johnston, 1998; Niewiadomska et al., 1999; Oda et al., 1997; Pacquelet and Rorth, 2005), and Cad99C, which regulates the length of microvilli, is essential for proper secretion of the vitelline membrane (D'Alterio et al., 2005; Schlichting et al., 2006). Here, we propose that Cad74A, a non-classical cadherin that differs structurally from classical cadherins, provides an additional link between eggshell patterning and morphogenesis.

Cad74A is one of 17 genes in the *Drosophila* genome encoding putative cadherin proteins and has 13 cadherin domains, a N-terminal signal peptide, a transmembrane domain, and a short uncharacterized

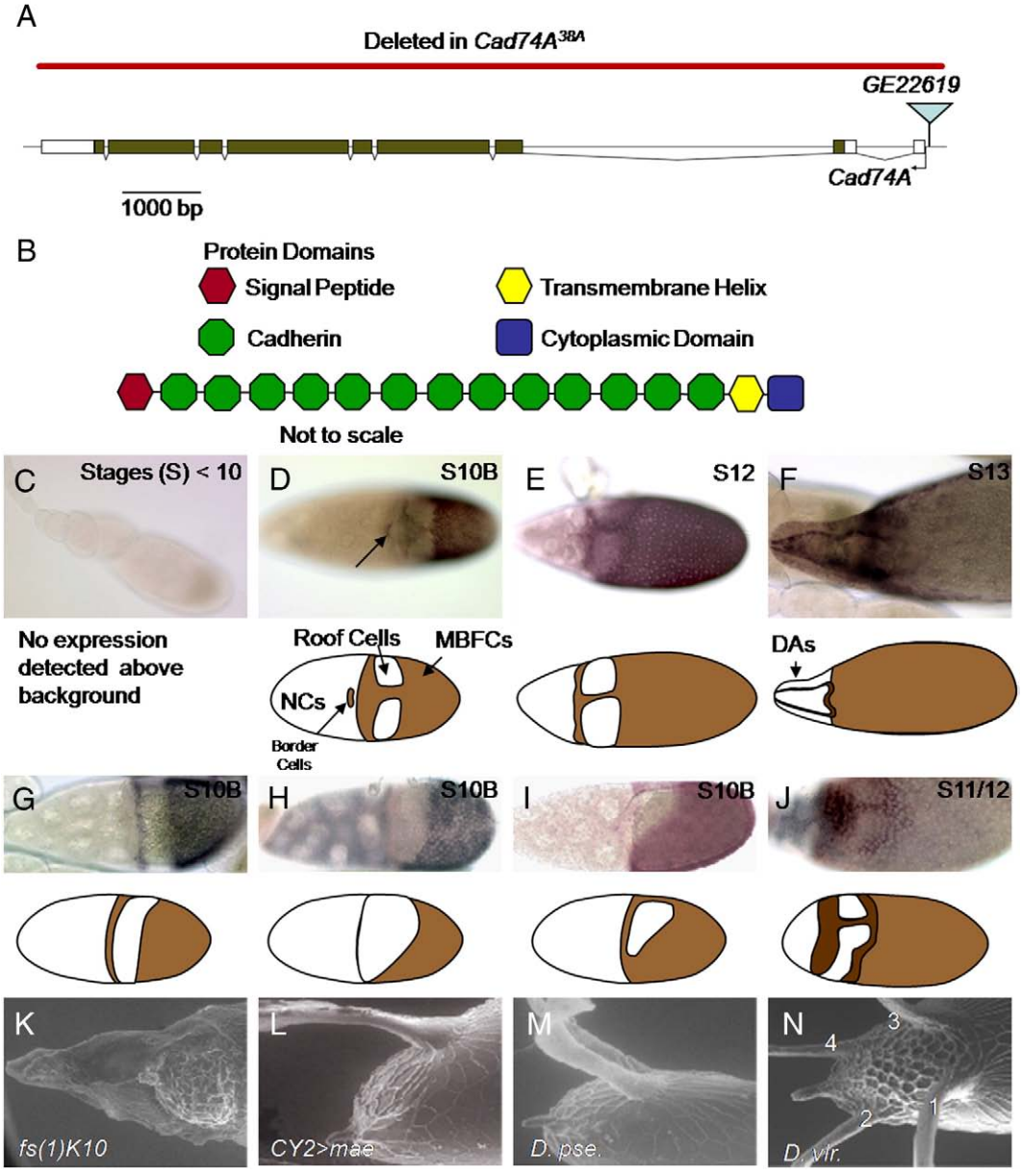


Fig. 1. Gene and protein architecture and expression of *Cad74A* during oogenesis. (A) The phenotypic analysis in this study was performed with *Cad74A*^{38A}, which has the entire coding region of *Cad74A* (on chromosome 3L) deleted. (B) Protein domain structure of Cad74A. Based on electronic annotation, Cad74A is a conserved putative cell adhesion molecule with 13 cadherin domains, an N-terminal signal peptide, a transmembrane domain, and a short uncharacterized cytoplasmic domain. (Hill et al., 2001; Hynes and Zhao, 2000). Putative cadherin domains were determined using the SMART tool (http://smart.embl-heidelberg.de) (Schultz et al., 1998). (C–F) Images of egg chambers after mRNA in situ hybridization. Cartoons highlighting the expression pattern are included below the images of egg chambers. (C) *Cad74A* transcript is not detected during early oogenesis. (D) Dorsal view of a stage 10 egg chamber; *Cad74A* is also expressed in the border cells (arrow) at the end of border cell migration. The following are marked on the cartoon: nurse cells (NCs), main body follicle cells (MBFCs), and roof cells. (E) Dorsal view of a stage 12 egg chamber. (F) Stage 13 egg chamber; *Cad74A* is strongly expressed in the apical region of the cells forming the respiratory appendages (dorsal appendages, DAs) and in the operculum domain. (G–J) In situ hybridization images of stage 10–11 egg chambers for *fs(1)K10* and *CY2>mae* genetic backgrounds and *D. pseudoobscura* (*D. pse.*) and *D. virilis* (*D. vir.*). (G) A late stage 10 *fs(1)K10* mutant background shows repression of *Cad74A* expression along an anterior lateral ring that extends to the ventral side. *Cad74A* is expressed along the dorsal midline and in the anteriormost follicle cells. (H) *Cad74A* expression in a stage 10 *CY2>mae* mutant background egg chamber. (I) *Cad74A* expression in stage 10 of *D. pse.* using the *D. mel. Cad74A* antisense probe. (J) *Cad74A* expression in *D. vir.* The patches of reduced *Cad74A* expression corresponds to the patches of BR protein reported for *D. virilis.* (Nakamura and Matsuno, 2003). (K–N) ESEM images of the ventral appendage sheath of a *fs(1)K10* egg (K), *CY2>mae* egg (L), a *D. pse.* egg (M), and the dorsal anterior egg structures for *D. vir.* with four DAs (N).

cytoplasmic domain (Hill et al., 2001; Hynes and Zhao, 2000) (Figs. 1A, B). A recent study that investigated the regulation of non-classical cadherins in the posterior spiracle reported that Cad74A mediates Ca-dependent homotypic cell–cell adhesion in cultured cells and is located sub-apically and apically in vivo (Lovegrove et al., 2006). *Cad74A* is also expressed in the early embryo, in the neurectoderm, the developing *Drosophila* larval brain, and eye imaginal disc, but the functional characterization of *Cad74A* has not been reported (Fung et al., 2008; Kearney et al., 2004; Schlichting and Dahmann, 2008; Tomancak et al., 2002).

Here, we report the dynamic expression of *Cad74A* mRNA and protein localization in oogenesis and show that this pattern correlates with the formation of multiple structural features of the eggshell. We demonstrate that the dynamic pattern of *Cad74A* expression is controlled by the EGFR and bone morphogenetic protein (BMP)/decapentaplegic (DPP) signaling pathways, two of the key regulators of follicle cell patterning (Berg, 2005). Specifically, high levels of BR, which is downstream of EGFR and BMP signaling, repress *Cad74A* in the roof cells while the Ets transcription factor, Pointed (PNT), activates *Cad74A* expression in the dorsal midline, likely by repressing BR (Deng and Bownes, 1997; Yakoby et al., 2008). Flies homozygous for an allele containing the complete deletion of *Cad74A* lay eggs that exhibit reproducible defects in the shape of the DAs, implying a possible role for *Cad74A* in eggshell morphogenesis. Strongly expressing *Cad74A* in the roof cells result in flattened, shortened appendages which are thickened at the base, suggesting that *Cad74A* has to be repressed in the floor cells to ensure proper DA elongation and migration. On the basis of these results, we propose that *Cad74A* is a component in the FC morphogenetic machinery that translates two-dimensional patterns into three-dimensional egg structures, possibly by modulating the adhesive properties of FCs.

Materials and methods

Fly stocks

The control stock used in this study is Oregon R (Ore R). GAL4 drivers and UAS lines that were used in this study include: *CY2>UAS-mae* (*edl*) (a gift from J. Duffy), *UAS-Broad-Z1* (Zhou et al., 2004), *UAS-pntP1* (Morimoto et al., 1996), *GR1-GAL4* (Gupta and Schupbach, 2003), *E4-GAL4* and *CY2-GAL4, UAS-λtop* (Queenan et al., 1997), *UAS-dnEGFR* (provided by A. Michelson), *UAS-Dad* (Tsuneizumi et al., 1997), *UAS-tkv** (Lecuit et al., 1996), and *br-GAL4* drivers were a gift of H. Cui and L. Riddiford. Since *CY2>pntP1* and *CY2>br-Z1* are lethal, the *UAS-pntP1* and *UAS-Broad-Z1* constructs were driven by the *GAL80-GAL4, CY2-GAL4* line (Maximiliano and Suster, 2004). To drive expression with the *GAL80* flies, the flies were incubated at 27 °C for 24 h. The *GAL80-GAL4, CY2-GAL4>UAS-Broad-Z1* flies were kept on yeast 12 h prior to dissection, and the *GAL80-GAL4, CY2-GAL4>UAS-pntP1* flies were put on yeast 24 h before dissection. *D. pseudoobscura* was provided by V. Orgogozo and D. Stern. We also used *D. virilis*, *rho2.2-lacZ* (Ip et al., 1992), *fs(1)K10* (Wieschaus et al., 1978), $Ras85D^{E62K}$ and $Ras85D^{05703}$ (Schnorr and Berg, 1996).

Clonal analysis

The FLP/FRT recombinant technique (Lee and Luo, 2001; Xu and Rubin, 1993) was used to generate loss of function clones where the standard protocol marks null clones with a loss of a GFP marker, and the MARCM technique marks the generation of clones with GFP expression. *Ras* null clones were generated using the following background: *e22c-Gal4 UAS-FLP; FRT82B* $Ras^{\delta C40b}$*/FRT82B ubi-GFP* (Hou et al., 1995; James et al., 2002). *br* clones, marked using the MARCM technique (Lee and Luo, 2001), were generated with the br^{npr-3} *FRT19A* line crossed to *P{tubP-GAL80}LL1 hsFLP FRT19A;;UAS-mCD8GFP tubGAL4/MKRS* flies (Ward et al., 2006). *cut* clones were generated with ct^{db7} *FRT18D/hGFP FRT18D; MKRS hsflp* (Blochlinger et al., 1988; Sun and Deng, 2005). The *br* and *ct* flies were heat shocked at 37 °C in an air incubator for 1–2 h, kept at 25 °C for 2–3 days (ct^{db7} flies) or 5 days (br^{npr-3} flies), and then fed during the final 24 h before dissection.

Cad74A lines

The entire coding region of *Cad74A* (coordinates 221107–209329 on AE003524) is deleted in $Cad74A^{38A}$ (coordinates of the deletion are 209199–221108 on AE003524), which was generated by an imprecise P-element excision of *GE22619* (GenExel, Inc) (Fig. 1A). GFP-tagged lines of *Cad74A* (*UAS-Cad74A-GFP53A*, *UAS-Cad74A-GFP99X*) were a gift of A. Jacinto (Lovegrove et al., 2006). The *UAS-Cad74ARNAi* line was obtained from the Vienna Drosophila RNAi Center (Dietzl et al., 2007).

In situ hybridization

A modification of the standard in situ hybridization protocol was used for mRNA localization experiments, which did not include the RNase treatment (Wang et al., 2006; Yakoby et al., 2008). Postfixation in 4% paraformaldehyde was done for 15 min and ovaries were incubated in prehybridization solution for 3 h at 60 °C. The *Cad74A* antisense probe was a gift from M. Halfon. The primary antibody used for the enzyme color reaction was mouse anti-Digoxigenin-AP (1:2000, Roche).

Antibody staining and imaging

Antibodies used include mouse anti-BR core (1:50, DSHB), Oregon Green phalloidin (1:1000, Molecular Probes), Hoechst dye to stain for nuclei (1:10,000), rat anti-DE-cadherin (DCAD2, 1:100; DSHB, Oda et al., 1997), and guinea pig anti-Cad99C (1:3000, gift of D. Godt). To generate the mouse polyclonal Cad74A antibody, a 10×Histidine-Tag recombinant peptide (1398–1750 aa) was used for immunization (PrimmBiotech). Secondary antibodies used were the Alexa Fluor and Oregon Green secondary antibodies (1:1000, Molecular Probes). A standard immunostaining protocol was followed with modifications (Laplante and Nilson, 2006). For double staining of the mouse anti-BR core antibody and the mouse anti-Cad74A antibody, the Zenon® Mouse IgG_1 Labeling Kit (Molecular Probes) was used at a 1:5 ratio of mouse anti-BR antibody to labeling reagent and then blocked with blocking reagent at a 1:6 ratio. The labeled antibody was then diluted 1:4 with PBS+0.2% Triton X-100. Incubation with the Zenon® complex was performed after the traditional immunostaining protocol for the polyclonal Cad74A antibody, followed by a 15 min fixation with 4% paraformaldehyde in solution with PBST (0.2% Triton). Confocal images were taken with a PerkinElmer RS3 Spinning Disk Confocal microscope, a Zeiss LSM 510 microscope, or a Nikon Eclipse E800 compound microscope. Images were processed and organized with ImageJ (Rasband, 1997–2006), Photoshop (Adobe Systems, Inc., San Jose, CA) or Picasa2 (Google, Mountain View, CA).

Results

Cad74A is expressed in the follicle cells contacting the oocyte during late oogenesis

Cad74A was one of the transcripts identified in a microarray-based search for new targets of EGFR and BMP pathways in the follicular epithelium (Yakoby et al., unpublished). As revealed by in situ hybridization staining, *Cad74A* is expressed in a dynamic pattern during late oogenesis (Figs. 1C–F). We did not detect *Cad74A* expression in earlier stage egg chambers (Fig. 1C). From stage 10 and onwards, *Cad74A* is expressed in all columnar follicle cells except in two dorsolateral patches. It is also expressed in the border cells, but

only after migrating from the anterior of the egg chamber to the oocyte/nurse cell boundary (arrow, Fig. 1D). Throughout the later stages of oogenesis, *Cad74A* expression is expressed in the floor cells of the DAs and in the main body follicle cells (MBFCs) (Figs. 1E, F).

Correlation between Cad74A expression and morphogenesis

Cad74A's ability to promotes homotypic cell-cell adhesion in transfected culture cells (Lovegrove et al., 2006) suggests a possible

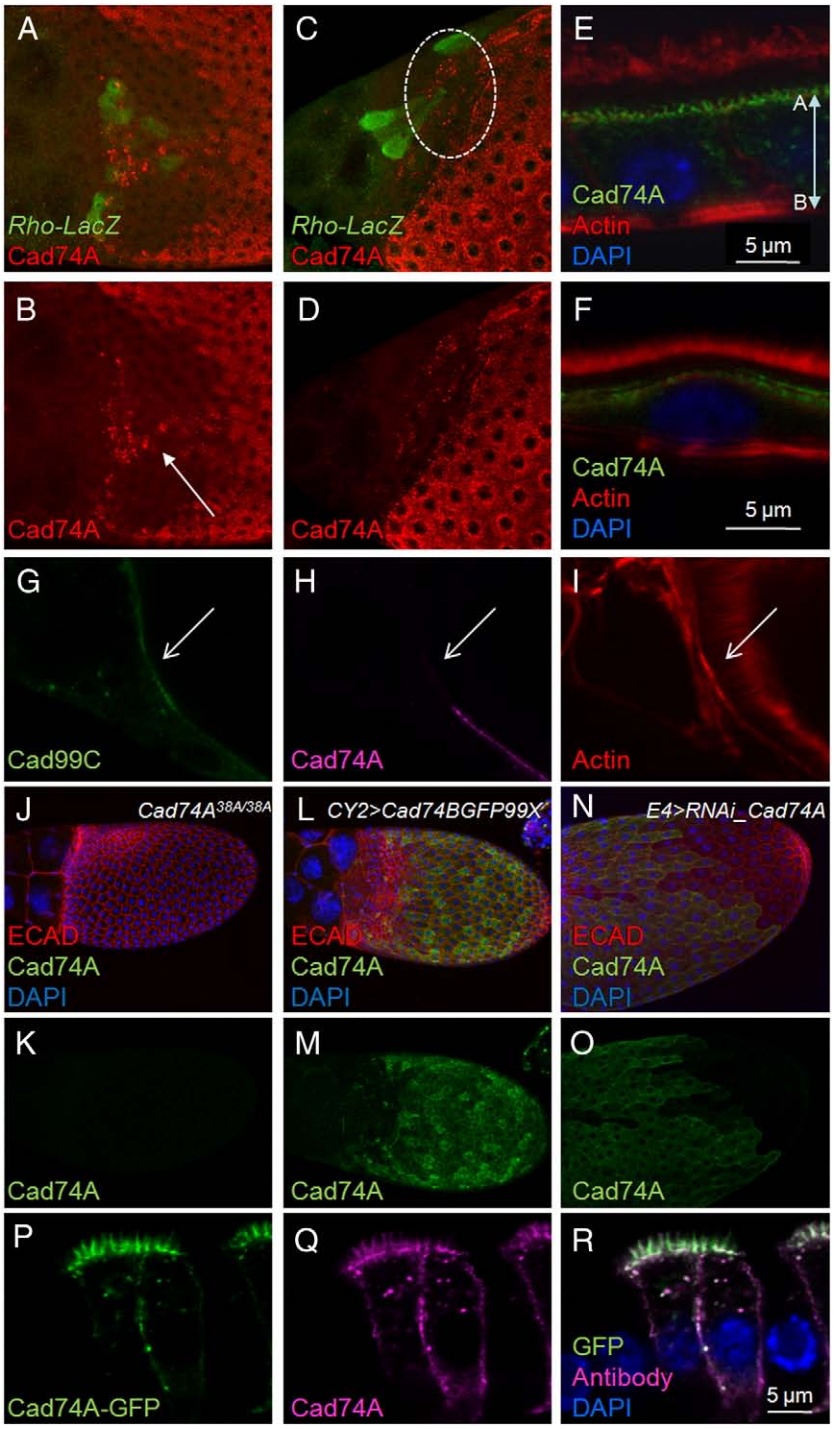


Fig. 2. Localization of Cad74A during oogenesis. (A, B) Immunostaining for Cad74A in a stage 11 egg chamber confirms the localization and expression of Cad74A along the apical surfaces and in the same expression pattern as the mRNA expression pattern with repression of protein in the roof cells. Cad74A is more punctate in dorsal midline and floor cells (marked by arrow) in a stage 11/12 egg chamber, cells expressing rho-LacZ (marked by green in panel A) provide a reference point for the location of the floor cells (Dorman et al., 2004). (C, D) Cad74A localization in a stage 12 egg chamber, lateral view. Punctate Cad74A is visible near the apices of the floor cells. (E) Localization of Cad74A in a magnified view of a single MBFC during mid-oogenesis (stage 10B). Cad74A is found in the microvilli and apical surface of the follicle cells (apical–basal axis indicated by arrow). (F) Cad74A remains localized to the apical surface at later stages in the MBFCs. The microvilli length is shorter in stage 12 MBFCs (a single magnified cell is shown). (G–I) At stage 12, Cad99C (G) and Cad74A (H) are at elevated levels in different cells. Phalloidin staining is brighter in cells with high levels of Cad99C (I). (J–M) No immunoreactivity to the Cad74A antibody is detected in Cad74A$^{38A/38A}$ egg chambers (J, K), in contrast to CY2>Cad74A-GFP egg chambers (L, M), which show strong Cad74A and GFP (not shown) staining in stage 11 MBFCs. (N, O) In *E4>Cad74ARNAi*, egg chambers, Cad74A staining is lost in the poster MBFCs, demonstrating the efficacy of the *UAS-Cad74ARNAi* line and the specificity of the Cad74A antibody. (P–R) Ectopic Cad74A-GFP expression, driven by the GR1-GAL4 driver in an early stage 10B egg chamber (P), is recognized by the Cad74A antibody (Q).

role for Cad74A in the morphogenesis of the dorsal appendages. One line of evidence that Cad74A plays a role in dorsal appendage formation is the correlation of the expression pattern in different genetic backgrounds with the final morphology of the dorsal anterior eggshell structures. For example, in *fs(1)K10* egg chambers, the mislocalization of Gurken, an EGF ligand secreted by the oocyte and required for FC patterning, gives rise to an eggshell with a broad ventral "veil" of dorsal appendage material (Wieschaus et al., 1978). Based on the proposed correlation between the *Cad74A* pattern and appendage morphology, we expected that the expression of *Cad74A* in this mutant would be repressed in the ventral anterior band of the follicle cells, as is indeed the case (Figs. 1G, K). In the *CY2>mae* background, which expresses the inhibitor of the transcription factor Pointed (Yamada et al., 2003) throughout the FCs using the GAL4/UAS system (Duffy, 2002), there is a single broad dorsal appendage (Fig. 1L). With the loss of the dorsal midline, the expression of *Cad74A* is repressed in a large dorsal patch of the follicular epithelium (Fig. 1H). Furthermore, repression of *Cad74A* in the roof cells of other species with varying number of DAs is conserved. This pattern holds for both *D. pseudoobscura,* which exhibits a similar *Cad74A* spatial pattern similar to *D. melanogaster* and has two appendages, and for *D. virilis*, which has two patches of *Cad74A* repression that corresponds to the shape of BR protein domain and has four appendages (James and Berg, 2003; Nakamura and Matsuno, 2003) (Figs. 1I, M, J, N). The conservation of the *Cad74A* pattern across species suggests a functional role in eggshell morphology. Moreover, *Cad74A* is repressed in cells that undergo apical constriction and bend out of the FC epithelial plane to form appendage material, suggesting the possibility that local repression of *Cad74A* is a necessary condition for cells to detach from the oocyte (allowing the floor cells to invaginate between the roof cells and the oocyte), an important step in the morphogenesis of DAs (Dorman et al., 2004). Given that Cad74A can promote homotypic cell adhesion (Lovegrove et al., 2006), repression of Cad74A in the roof cells may decreases adhesion between these cells, allowing increased junctional remodelling and, thus, arrangement between roof cells, a prerequisite for their morphogenetic movements away from the oocyte.

Cad74A localizes to the apical membranes of FCs

To determine the localization of the *Cad74A* protein, we raised a polyclonal antibody to Cad74A and found that *Cad74A* protein distribution largely mirrors the mRNA expression pattern. However, the distribution of Cad74A appears more punctate in the dorsal midline and floor cells (Figs. 2A, B). At later stages, when the medial row and the anterior row of floor cells appear to meet and fuse, Cad74A is located in a puncate pattern that surrounds the floor cell apices (Figs. 2C, D). In the MBFCs, Cad74A is enriched at the apical membrane, but cytoplasmic Cad74A is also detected, possibly representing internalized molecules (Figs. 2E, F). Cad74A strongly marks FC microvilli at stage 10B when protein is first detected. However, microvilli length decreases in the MBFCs during later stages of oogenesis. While Cad99C and Cad74A share similar staining to the apical membrane of FCs, the expression patterns of Cad74A and Cad99C differ dynamically as they do not appear to strongly co-localize in later stages of oogenesis (Figs. 2G–I).

Antibody immunoreactivity is specific to Cad74A as no staining is detected in $Cad74A^{38A/38A}$ egg chambers that were incubated in the same tube with eggs expressing the Cad74A-GFP construct, which served as an internal control. Co-staining for GFP allowed us to differentiate between the two genotypes (Figs. 2J–M). Furthermore, no immunoreactivity to Cad74A is found at late stages in the posterior FCs when *E4-GAL4* drives expression of a *Cad74A* RNAi construct (Dietzl et al., 2007; Queenan et al., 1997) (Figs. 2N, O), demonstrating the efficacy of the *UAS-Cad74ARNAi* line. When *Cad74A-GFP* was driven by the early driver, *GR1-GAL4* (Gupta and Schupbach, 2003), GFP and Cad74A staining co-localized to the same FCs (Figs. 2P–R), showing that the Cad74A antibody recognizes Cad74A-GFP. We did not detect any staining in the oocyte.

EGFR and BMP signaling jointly regulate Cad74A

The repression of *Cad74A* in the two dorsolateral patches suggests joint regulation by the EGFR and BMP signaling pathways, which have been shown to act as the key patterning signals of the dorsal anterior region of the follicular epithelium (Atkey et al., 2006; Berg, 2005; Dobens and Raftery, 2000; Goentoro et al., 2006; Shravage et al., 2007; Yakoby et al., 2008). Hence, we used the UAS/GAL4 system (Duffy, 2002) to perturb the EGFR and BMP pathways to test how *Cad74A* expression changes. When activated EGFR signals at high levels in the follicle cells (*CY2>λtop*), *Cad74A* is expressed in all of the main body follicle cells in stage 10 egg chambers except for the anteriormost row of cells (Fig. 3A). At later stages, the region of repression expands and then two dark bands of strong *Cad74A* expression are seen between a narrow band of repressed *Cad74A*, corresponding to a single band of high levels of BR (Figs. 3B, C, arrowhead). This band likely corresponds to the boundary between the operculum and the main body of the egg, which is shown in the dorsalized eggshell with no dorsal appendages (Fig. 3D). The broad, dark band of *Cad74A* expression matches the band of MAPK signaling seen in *CY2>λtop* as a result of the *rho* spatial pattern (not shown) (Queenan et al., 1997). In the opposite perturbation, downregulation of EGFR signaling (*CY2>dnEGFR*), results in a ventralized phenotype. In this background, *Cad74A* is expressed in all FCs, where uniform levels of BR are observed (Figs. 3E–H).

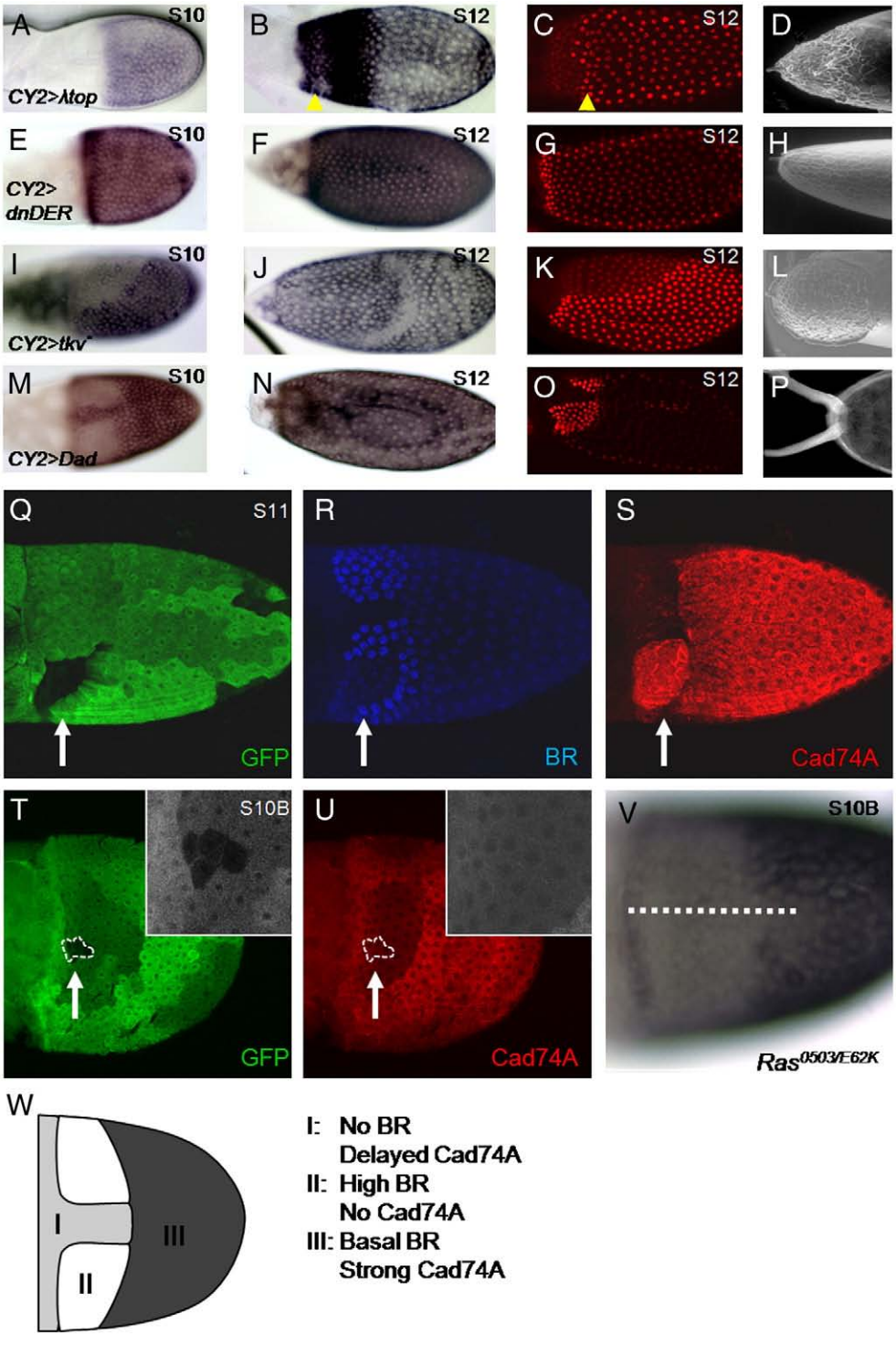
With ectopic expression of a constitutively active form of TKV receptor (*CY2>tkv**, Lecuit et al., 1996; Nellen et al., 1996), *Cad74A* is initially repressed throughout the dorsal anterior region (Fig. 3I). Later, the dorsal anterior region is filled with *Cad74A*, but a thin band in the shape of an arc remains (Fig. 3J). This band of *Cad74A* repression correlates to the band of high BR and may correspond to the boundary between the enlarged operculum and the main body of the egg (Figs. 3J–L). Ectopic expression of an inhibitor of BMP signaling (*CY2>Dad*) enlarges the roof cells towards the anterior direction (Yakoby et al., 2008), which is accompanied by repression of *Cad74A* in those cells (Fig. 3M). At later stages, the BR patches decrease in size and are shifted in the dorsal anterior direction (Figs. 3N–O). Consequently, the two dorsal appendages are also shifted toward the anterior edge of the egg chamber (Fig. 3P). In each of these genetic perturbations, we noticed that *Cad74A* is repressed in cells with high levels of BR, which suggests that high levels of BR may repress *Cad74A*.

Regulation of Cad74A by BR

To clarify the relationship between BR and *Cad74A,* we first examined cells homozygous for a null allele of *Ras*, a component of the EGFR signaling pathway. Previously it was shown that loss of *Ras* in the roof cells leads to a cell autonomous loss of BR (Atkey et al., 2006; Deng and Bownes, 1997; Yakoby et al., 2008). If BR represses *Cad74A*, then reduced levels of BR in $Ras^{\Delta C40b}$ clones should result in ectopic *Cad74A*. In 9/12 (75%) of stage 10B/11 egg chambers, we found this to be the case (Figs. 3Q–S). While BR is not eliminated from the patch, it is at a level comparable to the basal level found in the MBFCs, alleviating the repression of Cad74A by BR. Cad74A is found to be strongly expressed in such clones. The negative examples appeared in younger stage 10B egg chambers, suggesting that the loss of EGFR signaling and reduction of BR in the roof cells does not immediately induce Cad74A (Figs. 3T–U). On the other hand, downregulation of BR in the dorsal anterior requires high levels of EGFR signaling. Hypomorphic alleles of *Ras* would thus be expected to reduce *Cad74A* expression in the dorsal midline, which is what is observed (Fig. 3V). In each perturbation examined, we find changes in the shape

and location of three domains of *Cad74A* expression: I. Intermediate/delayed expression in the dorsal midline (High EGFR/BR is absent), II: Strong repression in the roof cells (Moderate levels of EGFR/high BR), III: strong expression in the ventral and posterior FCs (Low levels of EGFR/basal levels of BR) (Fig. 3W).

The appearance of *Cad74A* in the dorsal midline and the absence of *Cad74A* in the roof cells suggest the possibility that high levels of BR in the dorsal anterior FCs could be sufficient to repress *Cad74A* (Figs. 4A–C). The co-localization of basal BR and *Cad74A* in the posterior MBFCs could imply either that BR is not at high enough levels to repress Cad74A or acts as an activator at lower levels. To directly test the regulation of *Cad74A* by BR directly, we over-expressed the *br-Z1* isoform using the GAL80-GAL4, CY2-GAL4 system, which promotes expression in the MBFCs starting in mid-oogenesis (stages 8–10) (Queenan et al., 1997) when the flies are kept at elevated temperatures (see Materials and methods for details; Fig. 4D). Overexpression of *br* reduced levels of the *Cad74A* transcript in the dorsal anterior almost completely and to a lesser extent in the posterior and ventral FCs (Figs. 4E, F). Because the repression of *Cad74A* by BR was mostly confined to the dorsal anterior region of the FCs, we examined the BR protein distribution in this background. Interestingly, we found that the highest levels of BR in the *CY2>br-Z1*



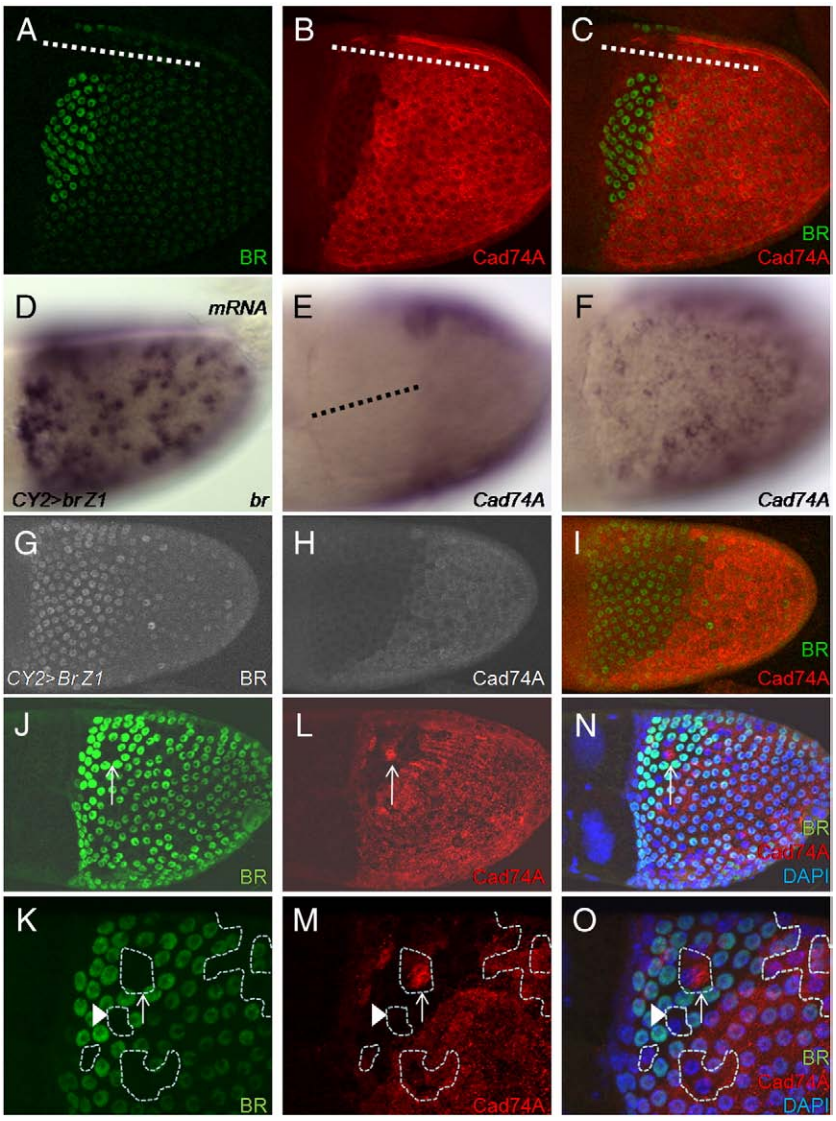


Fig. 4. BR represses Cad74A in the roof cells. (A–C) The high level of BR in the roof cells (A), which correlates with Cad74A repression in those cells (B), as seen in the merged image (C), suggests the possibility that BR represses Cad74A. (D–F) Overexpression of *br-Z1* in *CY2>br-Z1* (D) often leads to a loss of Cad74A in the dorsal anterior bridge (E) and more patchy expression in the posterior and ventral regions of the egg chamber (F). (G–I) BR-Z1 is more highly expressed in the anterior, even when driven by the driver *CY2-GAL4*, which drives patchy expression throughout the MBFCs (G, I), resulting in a loss of Cad74A in the dorsal anterior. (J–O) In mosaic egg chambers, clones homozygous for the null allele $br^{npr\text{-}3}$, marked by BR (J), result in relieved repression of Cad74A in the roof cells (L, N). Panels K, M, O are magnified images of the roof cells shown in panels J, L, N.

background were limited mostly to the anterior, with patchy up-regulation of BR in the posterior main body as shown for egg chambers double stained for BR protein and Cad74A, demonstrating not only that Cad74A is negatively regulated by ectopic BR, but also a possibility that BR may undergo post-transcriptional autoregulation (Figs. 4G–I).

Fig. 3. *Cad74A* expression is regulated by the EGFR and BMP signaling pathways. (A) In *CY2>λtop* (upregulation of EGFR signaling in all MBFCs), *Cad74A* expression is uniform in the follicle cells overlaying the oocyte except for the anteriormost row of follicle cells. (B) At later stages, the repression seen in the anterior region is replaced with a dark band of expression, followed by a band of repression (arrowhead), and a second band of dark expression. The band of repression likely corresponds to the boundary of operculum material. (C) BR protein staining with slightly brighter staining in a band (arrowhead) that may correspond to *Cad74A* repression in panel B (arrowhead). (D) In *CY2>λtop*, The eggshell morphology is completely dorsalized so no dorsal appendages, which are normally dorsolateral, form. (E) In *CY2>dnEGFR* (down regulation of EGFR), *Cad74A* is expressed uniformly as well. The anteriormost row of follicle cells is stained more darkly. (F) Uniform expression continues in later egg chambers. (G) BR staining in stage 12 *CY2>dnEGFR*. (H) In *CY2>dnEGFR*, the eggshell is ventralized. (I) In *CY2>tkv** (ectopic BMP signaling), the dorsal anterior expression of *Cad74A* is repressed, which corresponds to the loss of dorsal appendages and increased operculum seen in the final eggshell morphology. (J) At later stages, a band of *Cad74A* repression is clearly seen, corresponding to the operculum boundary shown in panel L. (K) BR staining in stage 12 *CY2>tkv** egg chamber. (L) In *CY2>tkv**, the dorsal appendages are absent and the operculum is enlarged. (M) In *CY2>Dad* (downregulation of BMP signaling), the patches of *Cad74A* repression are larger at earlier stages. (N) In a stage 12 egg chamber the patches of repression are smaller and shifted in the anterior direction. (O) BR staining in a different stage 12 egg chamber. (P) An anterior shift in the dorsal appendages is seen in a *CY2>Dad* egg. (Q–S) Complete loss of EGFR signaling in the roof cells homozygous for the null *Ras* allele ($Ras^{\Delta C40b}$) reduces BR to a basal level equivalent to the level in the posterior MBFCs. Cad74A is ectopically expressed in the roof cells (marked by an arrow). (T, U) An earlier *Ras* clone located in the roof cells in a stage 10B egg chamber (T) does not show significant ectopic Cad74A (U). (V) In the hypomorph *Ras* background $Ras^{0503/E62K}$, *Cad74A* transcript is lost in the midline due to the loss of PNT and ectopic BR (not shown) in the dorsal in the dorsal anterior bridge. Here, and in other figures, the dotted line marks the dorsal midline. (W) Summary of *Cad74A* expression and BR levels in three different regions of the follicular epithelium which is maintained across the various examined perturbations in EGFR and BMP signaling: Region I (dorsal midline): No BR staining is detected, and *Cad74A* expression is slightly delayed and at lower levels. This effect is most apparent in *CY2>tkv** egg chambers, but is also found in WT (Figs. 1D, 2A, B). Region II: High BR levels in the roof cells and no *Cad74A*. Region III: Basal levels of BR and strong *Cad74A* expression.

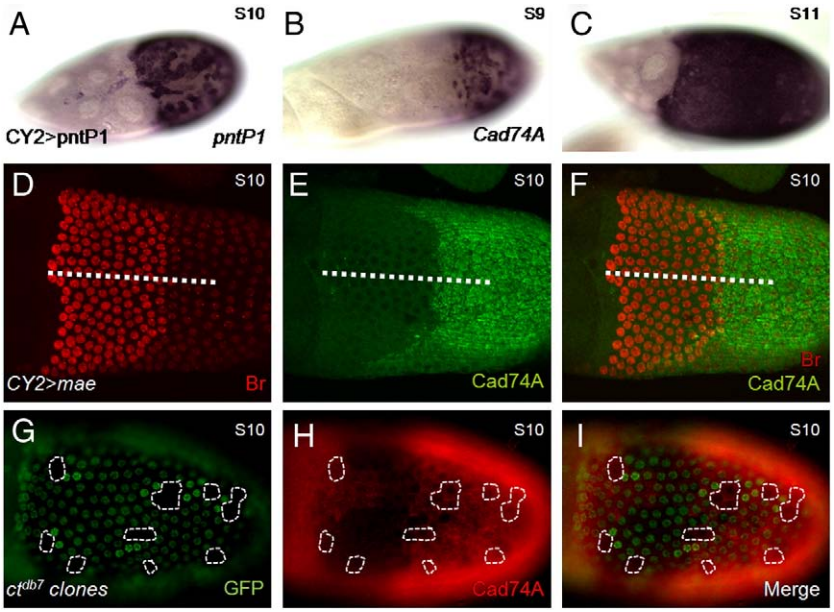


Fig. 5. Regulation of *Cad74A* by other transcription factors. (A) Ectopic expression of the transcription factor *pntP1* (*CY2>pntP1*) results in ectopic and early expression of *Cad74A* (B) which overcomes repression of *Cad74A* in the roof cells (C). (D–F) Loss of PNT activity in the *CY2>Mae* background results in ectopic BR in the dorsal midline (D) accompanied by a loss of Cad74A (E). (G–I) Examination of Cad74A expression in numerous ct^{db7} clones, marked by the loss of nuclear GFP (G), suggests that *Cad74A* is not regulated by the transcription factor Cut (H, I).

To confirm that BR is both necessary and sufficient for repressing *Cad74A* in the roof cells, we examined Cad74A expression in egg chambers mosaic for the null allele, $br^{npr\text{-}3}$ (Belyaeva et al., 1980; Kiss et al., 1988) using the MARCM technique (Lee and Luo, 2001). As noticed earlier (Ward et al., 2006), loss of BR was not faithfully marked by GFP expression, although the presence of GFP, which was found in a minority of cases, accurately marked homozygous null $br^{npr\text{-}3}$ clones. We therefore marked clones with a loss of BR staining. The tilt and keystone shape of the roof cells, as well as the invagination of *Cad74A*-expressing floor cells made it difficult to determine whether ectopic Cad74A expression in the roof cells was completely cell autonomous. To avoid confusing the invaginating *Cad74A*-expressing floor cells with ectopic expression in $br^{npr\text{-}3}$ clones, we focused mainly on scoring clones in the roof cells of stage 10B/11 egg chambers. We found ectopic Cad74A expression in 94% (30/32 distinct clones, 20 egg chambers examined) of clones examined in the roof cells, of which 75% (24/32 clones) appeared to be completely cell autonomous (Figs. 4J–O). In two clones, we did not see expression of Cad74A (both stage 10B egg chambers, one example is shown (arrowhead) in Figs. 4L, M) and in six additional clones, the ectopic expression in the clone did not appear to be span the clone completely. We thus conclude that high levels of BR repress Cad74A in the roof cells, but that other factor(s), including delayed induction or repression independent of BR, result in cases where *Cad74A* is still repressed in $br^{npr\text{-}3}$ homozygous cells during stages 10B/11.

Regulation of Cad74A by additional transcriptional factors

Based on the difference in the levels of Cad74A expression in the midline and the main body, we examined the role of *pointed* (*pnt*) in regulating Cad74A. Downregulation of BR in the dorsal anterior requires the Ets transcription factor *pnt*, which is in turn expressed only at the highest levels of EGFR signaling (Deng and Bownes, 1997; Morimoto et al., 1996; Yamada et al., 2003). To confirm the regulatory connection between *pnt* and *Cad74A*, the *pntP1* isoform was ectopically expressed starting at stage 8, which resulted in the early (earlier than stage 10) and strong expression of *Cad74A* throughout the main body FC epithelium and the loss of repression of Cad74A in the roof cells (Figs. 5A–C). In the opposite direction, ectopic expression of *Mae*, an inhibitor of *pntP2* transcriptional activity (Yamada et al., 2003), leads to a loss of *Cad74A* in the dorsal anterior bridge, demonstrating that PNT, likely through repression of BR in the dorsal anterior, is required for *Cad74A* expression (Figs. 5D–F).

The regulation of *Cad74A* changes mainly in the dorsal anterior domain when perturbations in either EGFR or BMP signaling are made, implying that other inputs may govern *Cad74A* regulation in the ventral and posterior side of the egg chamber. *Cad74A* was shown to be downstream of *cut* in the posterior spiracle (Lovegrove et al., 2006). The transcription factor *cut* is involved in cell-cycle progression and FC differentiation in early stages of oogenesis and later reappears at stage 10B (Sun and Deng, 2005), when *Cad74A* is first expressed. We examined egg chambers mosaic for the null allele *cut*, ct^{db7} (Blochlinger et al., 1988) and found that expression of *Cad74A* is unaffected in 100% of cut^{db7} clones examined (10/10 egg chambers) (Figs. 5G–I). This result suggests that different enhancers are responsible for expression in the FCs and the posterior spiracle.

Cad74A is required for robust DA formation

To test the hypothesis that *Cad74A* is functionally important for DA formation, we generated a null allele, $Cad74A^{38A}$, using imprecise P-element excision that resulted in the complete deletion of the gene with no *Cad74A* transcript detected (Fig. 1A; Figs. 6A, B).

Homozygous $Cad74A^{38A}$ flies reproducibly lay a subset of eggs with severe DA defects. We found that 17% (315/1820 eggs examined over multiple independent counts) of $Cad74A^{38A/38A}$ eggs laid at room temperature lacked appendages or exhibited appendages that are significantly deformed or shortened to varying degrees (Figs. 6C–F).

As a control, the parental line used to generate the deletion does not show loss of DAs (data not shown). In the fraction of $Cad74A^{38A/38A}$ eggs with severely disrupted DAs, a range of DA length was observed, suggesting that tube elongation, a major step in eggshell morphogenesis (Berg, 2005; Dorman et al., 2004), was affected. We did not see signs of collapsed eggs, confirming the observation based on localization that Cad99C (D'Alterio et al., 2005; Schlichting et al., 2006) and

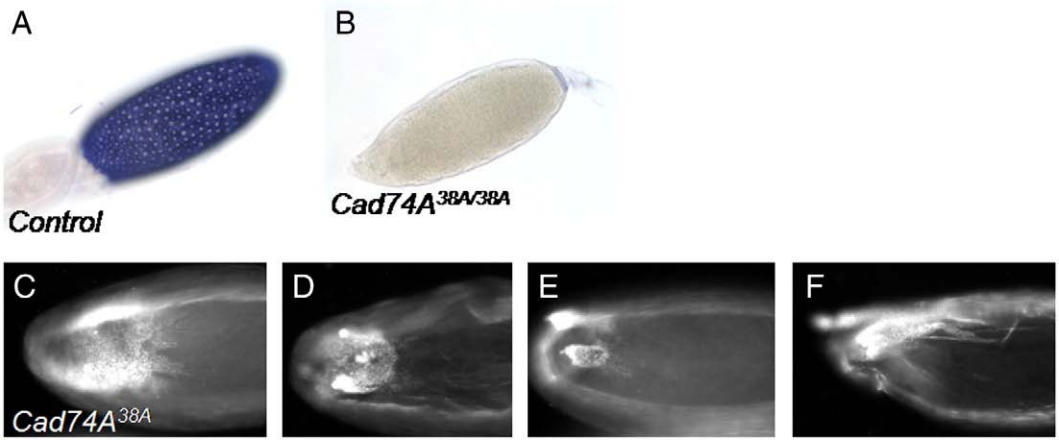


Fig. 6. Phenotypic analysis of the *Cad74A*^{38A} allele. (A, B) In situ hybridization of *Cad74A* in control egg chambers (A) is completely absent in homozygous *Cad74A*^{38A} egg chambers (B). (C–F) Severe DA phenotypes in homozygous *Cad74A*^{38A} egg chambers range from a complete loss of DAs (C) to DAs of varying lengths not seen in the control parental line (D–F).

Cad74A have different functions. To verify this conclusion, we investigated the permeability of *Cad74A*$^{38A/38A}$ eggs after chemically removing the outer chorion to the dye Neutral Red (Schlichting et al., 2006) and found that *Cad74A*$^{38A/38A}$ eggs were impermeable to Neutral Red (data not shown), as expected if the vitelline membrane was deposited correctly.

Ectopic expression of Cad74A in the roof cells hinders DA elongation

To test our hypothesis that repression of Cad74A in the roof cells is important for DA formation, we expressed *Cad74A-GFP* (*UAS-Cad74A-GFP53A*) in roof cells using a late stage *br-GAL4* driver developed in the Riddiford lab. Overexpression with *br>Cad74A-GFP* gave a strong phenotype with the DAs largely replaced by thick, flattened ridges (Fig. 7B), compared to *br>GFP* eggs with properly formed DAs (Fig. 7A) and eggs laid by sister flies to *br>Cad74A-GFP* (not shown). While the controls also sometimes exhibited shortened DAs, the phenotype was always less penetrant and less severe (Fig. 7C). The strong expression of Cad74A-GFP in the roof cells results in large aggregates of Cad74A in the apical half of the roof follicle cells (Figs. 7D–F). Apical constriction was sometimes affected in *br>Cad74A-GFP* egg chambers during stage 12, but not absolutely prevented (Figs. 7G–N). In egg chambers at late stage 13/14, the roof cells marked by BR (Fig. 7R compared to the control, Fig. 7O) failed to complete migration towards the anterior tip of the egg chamber, consistent with the observed eggshell phenotypes (Fig. 7B). Based on these overexpression experiments, we suggest that repression of Cad74A in the roof cells is required for robust DA morphogenesis.

Discussion

High levels of BR repress Cad74A in the roof cells

Starting at stage 10, *Cad74A* is expressed in the main body FCs, except for cells expressing high levels of BR, corresponding to the roof primordia. As oogenesis proceeds, *Cad74A* continues to be expressed in the cells surrounding the roof cells, which eventually detach from the oocyte as the floor cells slip between the roof cells and oocyte. The dynamic expression pattern of *Cad74A* and its ability to promote cell adhesion (Lovegrove et al., 2006) suggests that *Cad74A* is a link between FC patterning and morphogenesis.

While investigating how the EGFR and BMP signaling pathways jointly regulate *Cad74A* expression, we found that perturbations in either pathway result in transitions of *Cad74A* expression that can be predicted from the final DA morphology or the corresponding expression pattern of high levels of BR. Based on the expression pattern of *Cad74A* and BR, two general models of regulation can be envisioned (Figs. 8A, B):

1) *Cad74A* is uniformly activated throughout the follicular epithelium from stage 10 and throughout late oogenesis and is repressed in the roof cells by sufficiently high levels of BR, or

2) *Cad74A* is uniformly activated at low levels of EGFR signaling in the ventral and posterior MBFCs and repressed by an unknown factor expressed in the dorsal anterior. A second signal in the dorsal midline and floor cells activates *Cad74A*, which is slightly delayed from ventral and posterior expression. In this model, BR and *Cad74A* are regulated in parallel.

BR has long served as a marker for DA morphogenesis (Deng and Bownes, 1997; Dorman et al., 2004; Tzolovsky et al., 1999) and is a conserved regulator of morphogenesis during many other stages such as pupation (Konopova and Jindra, 2008; Suzuki et al., 2008). Through ectopic expression and loss-of-function mosaic analysis, we show that the repression of *Cad74A* in the roof cells is mediated by BR (consistent with model 1). The basal level of BR in the posterior and ventral MBFCs appears to only slightly repress *Cad74A* (data not shown), suggesting that another activator is present to stimulate *Cad74A* expression in the MBFCs or that the basal level of BR is not sufficient to repress *Cad74A*. Additionally, *Cad74A* is also activated by the Ets transcription factor PNT, possibly indirectly through repression of BR (Deng and Bownes, 1997). In conclusion, while the first model of regulation (uniform activation – high levels of BR) appears to be involved, the second possible mode of regulating *Cad74A* (uniform activation – dorsal repression + a second dorsal midline signal) cannot be ruled out because: 1) *Cad74A* in the posterior and ventral MBFCs is expressed at stronger levels and slightly earlier to the dorsal midline expression, 2) PNT acts as an effective ectopic activator of *Cad74A* expression even before stage 10, and 3) loss of BR in *Ras* and *br* clones does not absolutely ensure *Cad74A* expression at stages when *Cad74A* is expressed in the other FCs. Finally, we examined a known factor regulating *Cad74A* during embryogenesis, *cut*, which is expressed uniformly in the FCs during mid-oogenesis (Sun and Deng, 2005), and found no effect in the main body FCs. Future work will require identifying the specific transcription factors that stimulate *Cad74A* expression in the posterior and ventral MBFCs. Establishing the regulatory connection between BR and *Cad74A* places *Cad74A* into the larger regulatory network responsible for patterning the FCs during mid-oogenesis (Yakoby et al., 2008; shown in Fig. 8C).

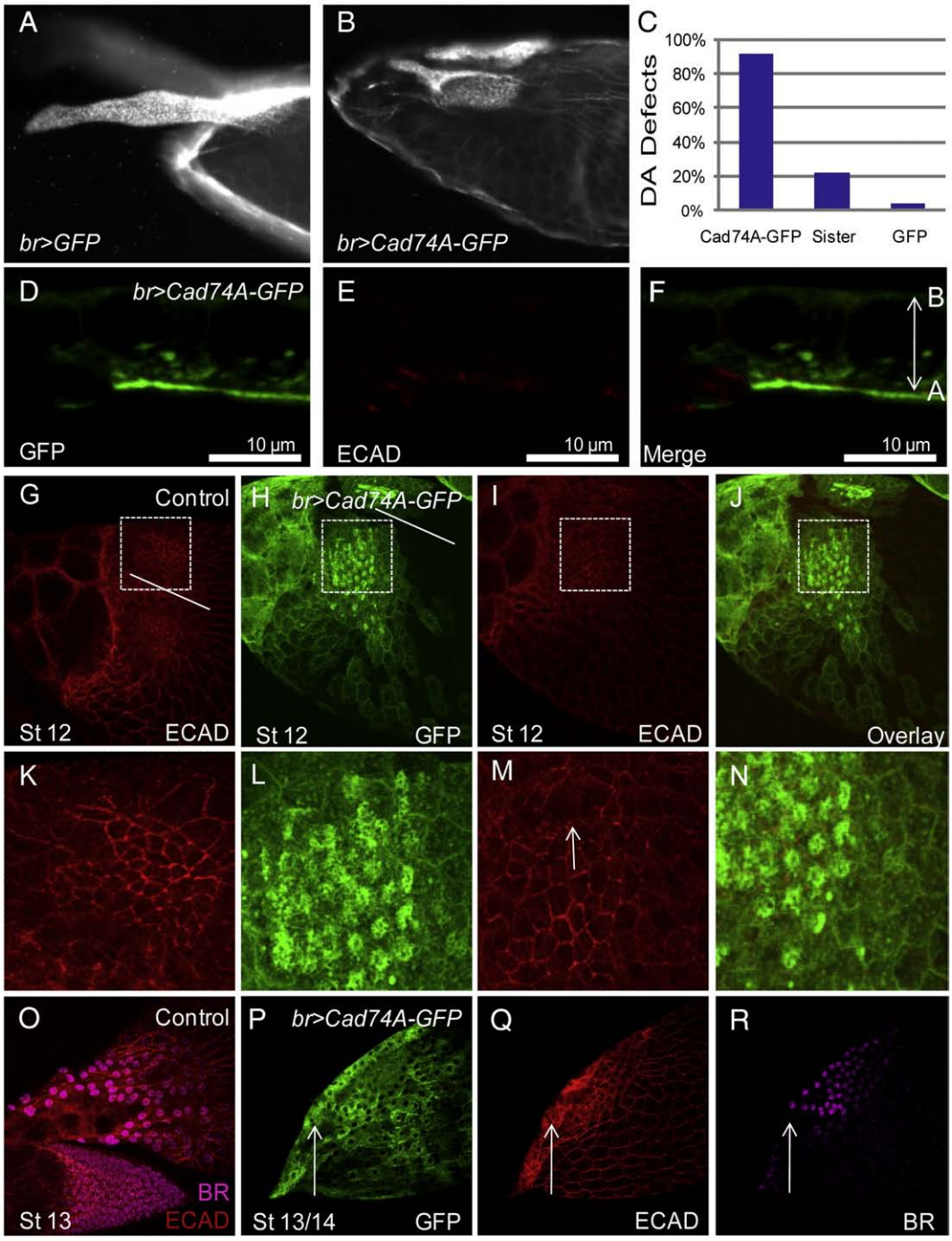


Fig. 7. Overexpression of Cad74A-GFP hinders the proper migration of the roof cells. (A) DAs of *br>GFP* eggs project out of the main body of the egg. (B) Shortened, flattened DAs in a *br>Cad74A-GFP53A* egg. (C) The *br>Cad74A-GFP53A* flies lay eggs with a high penetrance of flattened, short DAs (285/308 eggs scored, 93%) compared to the sister flies (*br-GAL4/TM6C, 42/188, 22%*) or *br>GFP* (2/51, 4%) flies. (D–F) *br-GAL4* strongly drives *UAS-Cad74A-GFP53A* in the roof cells, resulting in large aggregates of Cad74A in the apical half of the roof follicle cells. (G–N) Compared to control sister flies (G), *br>Cad74A-GFP53A* egg chambers at stage 11/12 show defects in apical constriction, accompanied by the formation of large aggregate bodies (H–J). The slanted lines mark the dorsal midline. Panels K–N are magnified images of the roof cells, the disruption in apical constriction is indicated by an arrow in panel M. (O–R) Compared to control sister flies (O), migration of the roof cells is severely affected in late stage 13/14 *br>Cad74A-GFP53A* eggs (P–R). Arrows in panels P–R mark the anterior extent of roof cell migration, as marked by BR in panel R.

The role of Cad74A in epithelial morphogenesis

Previous efforts to genetically probe for the function of non-classical cadherins in the posterior spiracle, including Cad74A, Cad88C, Cad86C, and Cad96C, have been confounded by a lack of a strong phenotype and functional redundancy (Lovegrove et al., 2006). This was also found to be the case for Cad86C, which is specifically upregulated in cells of the morphogenetic furrow of the eye disc, but does not show a phenotype in the eye imaginal disc (Schlichting and Dahmann, 2008). The lack of a phenotype in single knock-downs confirms the robustness of the morphogenetic program to a loss of expression of many effector proteins. The redundant function of the non-classical cadherins in the posterior spiracle suggests that single loss-of-function alleles of *Cad74A* should not lead to strong phenotypes. To test this expectation for *Cad74A* in oogenesis, we created and analyzed a complete null of *Cad74A* (*Cad74A*^{38A}). Surprisingly, *Cad74A* does not appear to be fully redundant with other adhesion molecules as complete deletion of *Cad74A* leads to partially penetrant, but severe

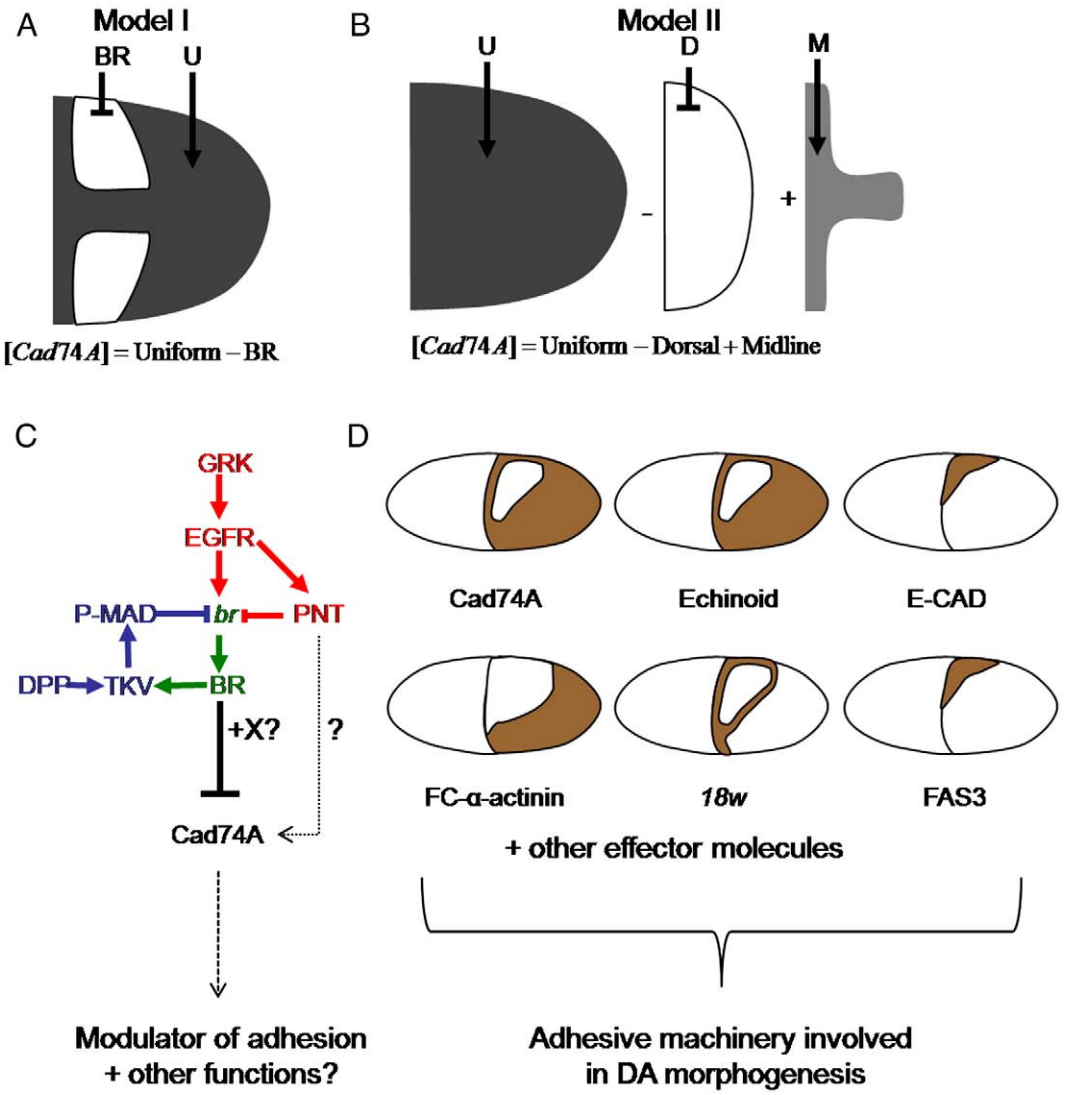


Fig. 8. From patterns to gene regulation and morphogenesis of effector proteins. (A) In model I, *Cad74A* is activated by a uniform signal starting at stage 10 and repressed by BR in the roof cells. (B) In an alternate model, *Cad74A* expression requires a uniform signal, repression by an unknown dorsal TF, and activation by a midline activator (such as PNT). (C) Regulation of *Cad74A* by the EGFR and BMP pathways based on the results of BR regulating *Cad74A* (this work) and previously reported research (Yakoby et al., 2008). Based on ectopic expression of *Cad74A* in the roof cells, we propose that Cad74A mediates adhesion to the oocyte, but do not rule out other functions such as secretion of the chorion. (D) Expression patterns for several effector genes and proteins at stage 10B, including: *Cad74A* (this work), Echinoid (ED) (Laplante and Nilson, 2006), basal E-CAD (James et al., 2002), FC-α-actinin (Wahlstrom et al., 2006), *18w* (Kleve et al., 2006) and FAS3 (Ward and Berg, 2005) possibly are regulated by BR, either directly or indirectly, in a synergistic and coordinated manner to ensure robust eggshell morphogenesis and the formation of the DAs.

DA phenotypes, where the migration and tubular elongation of the DAs is severely disrupted.

The disruption of the formation of the DAs suggests that the morphogenesis and migration of the roof cells is compromised. This may be due to the loss of segregation between the roof and surrounding follicle cells due to differences in adhesion between the follicle cells or a change in the surface tension along the apical membrane. Alternatively, Cad74A may play a role in epithelial polarity or in the secretion of the chorion. Overexpression of Cad74A specifically in the roof cells affects cell migration, consistent with a hypothesis that Cad74A acts as a "glue" to the oocyte. Alternatively, ectopic Cad74A in the roof cells prevents remodeling of the adherens junctions during intercalation (Dorman et al., 2004). Down regulation of Cad74A in ectopic patches is not sufficient to cause the FCs to detach from the oocyte as evident in the overall normal FC morphology found in posterior follicle cells when Cad74A was knocked-down (*E4>Cad74ARNai* background, Figs. 2N–O). As a preliminary conclusion, we suggest that Cad74A could perform a role in modulating the surface tension along the apical domain or in stabilizing cell–cell connections, but this model will require confirmation through future biochemical studies that identify the interacting partners of Cad74A. Finally, the incomplete penetrance of the *Cad74A* null allele may be a function either of a partial functional redundancy with other adhesive molecules (a strong possibility due to the intricate patterning of multiple adhesive molecules in this tissue) or due to the inherent robustness of morphogenesis (the machinery that performs the unit operation of cellular changes compensates if one aspect is adversely affected) (Kafri et al., 2006). A comprehensive study of double knock-downs of adhesive molecules will shed light onto this problem in the future.

Syn-expression groups of genes involved in morphogenesis

Producing an atlas of differentially expressed effector genes will be important to identify the roles of adhesion and cytoskeleton genes in the morphogenesis of the DAs. *Cad74A* shares a similar two-dimensional expression pattern with several other documented effector genes, and thus the model for *Cad74A* regulation can serve

as a backbone to compare and contrast the regulation of these other effector genes. For example, the *Cad74A* mRNA expression pattern during stage 10B is strikingly similar to the expression of Echinoid (ED) protein, a cell adhesion molecule which is implicated in the formation of an actomyosin contraction cable that surrounds the roof cell primordia boundary, with an important difference: expression in the dorsal midline appears later in Cad74A but earlier for ED (Laplante and Nilson; Fig. 8D). The homophilic adhesion molecule Fasciclin 3 (FAS3) also accumulates in the dorsal anterior region of the egg chamber and likely plays a role in setting the boundary between roof and floor cells (Ward and Berg, 2005). A basal pool of DE-Cadherin, separate from apical DE-Cadherin, is found at elevated levels in the dorsal anterior cells (James et al., 2002). The toll-like receptor, *18-wheeler* (*18w*), which may also play a role in adhesion or cell signaling by regulating the Rho-GTPase-signaling pathway (Kolesnikov and Beckendorf, 2007), is also expressed in a ring that appears to surround the roof cells during mid-oogenesis (Kleve et al., 2006). A subpopulation of α-actinin, which is involved in cross-linking and bundling actin filaments, is also expressed in the somatic FCs but repressed in the dorsal anterior follicle cells during mid-oogenesis and only overlaps BR in the posterior edge of the roof primordia (Wahlstrom et al., 2006). Thus, we group *Cad74A* with *Ed*, *Fas3*, *E-Cad*, *18w* and *FC-α-actinin*, as effector genes that show absence, reduction, or differential expression in the roof cell primordia compared to the surrounding cells (Fig. 8D). This does not imply that these effector proteins are interacting directly, but that expression of high levels of these effector proteins in overlapping patterns is required to coordinate the morphogenesis of the DAs. The differences in the expression of these effector genes between the dorsal midline (on one side of the roof cell boundary) and the posterior and ventral MBFCs (the other side of the roof cell boundary) may reflect differences in the two possible modes of regulation discussed previously.

The correlation between final dorsal appendage morphology and *Cad74A* expression suggests that the regulation of genes involved in DA formation is highly modular and interconnected (e.g., a shift in BR expression corresponds to a shift in the domain of *Cad74A* repression that correlates with final dorsal appendage morphology). Further characterization of the regulation and function of *Cad74A* and other effector genes during epithelial morphogenesis will be important in synthesizing a comprehensive mechanism of dorsal appendage formation.

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